ISOLEUCINE AND VALINE METABOLISM IN *ESCHERICHIA COLI*

XI. VALINE INHIBITION OF THE GROWTH OF *ESCHERICHIA COLI* STRAIN K-12[1]

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ABSTRACT

LEAVITT, RICHARD I. (Harvard Medical School, Boston, Mass.) AND H. E. UMBARGER. Isoleucine and valine metabolism in *Escherichia coli*. XI. Valine inhibition of the growth of *Escherichia coli* strain K-12. J. Bacteriol. **83**:624–630. 1962.—The inhibition of the growth of *Escherichia coli* strain K-12 by valine was shown to be due to the sensitivity of the acetohydroxybutyrate-forming system to valine. It was demonstrated that both *E. coli* strain W, a strain whose growth is unaffected by valine, and a valine-resistant mutant of strain K-12 have acetolactate- and acetohydroxybutyrate-forming systems which are less sensitive to valine than that of strain K-12. It was further shown that α-aminobutyrate accumulates in the culture fluid of the valine-sensitive strain when incubated in the presence of valine. The levels of valine in the "free amino acid pool" were examined and found to be related to the differences in valine sensitivity of the acetolactate-forming systems of the three strains.

In one of the earliest studies of amino acid imbalance in bacterial nutrition, Gladstone (1939) observed that the growth of *Bacillus anthracis* was prevented by the addition of any one of the three branched-chain amino acids to a synthetic medium. It was suggested at that time that an excess of one amino acid might block either the incorporation or the synthesis of structurally similar amino acids. A similar interaction was later cited by Tatum (1946), who observed that valine inhibited the growth of the K-12 strain of *Escherichia coli* and that isoleucine reversed this inhibition. A later analysis of this inhibition served to focus attention on the biosynthesis of isoleucine rather than its incorporation as the probable site of inhibition by valine (Umbarger and Brown, 1955). The evidence included the fact that isoleucine was a noncompetitive antagonist of valine and that the only compounds known to reverse the inhibition were isoleucine and its two six-carbon precursors then known, α-keto-β-methylvalerate and α-,β-dihydroxy-β-methylvalerate. A four-carbon precursor, α-ketobutyrate, was ineffective. Since the enzymes catalyzing the two known reactions in the valine pathway were also essential enzymes in the isoleucine pathway, it was suggested that valine is inhibitory to *E. coli* K-12 because the enzyme over which valine exerts feedback control is also essential in isoleucine biosynthesis.

With the identification of the enzyme forming acetolactate as the site at which valine exerts feedback control (Umbarger and Brown, 1958), a more precise examination of the inhibitory action of valine seemed possible. Unfortunately, this examination could not be undertaken until a specific method for the determination of acetohydroxybutyrate was developed. The recent development of such an assay (Leavitt and Umbarger, 1960) has made it possible to obtain evidence that acetohydroxybutyrate and acetolactate are formed by the same enzyme system (Leavitt and Umbarger, 1961).

In this paper an examination of the inhibition of acetohydroxybutyrate formation by valine in *E. coli* strains W, K-12, and a valine-resistant mutant of strain K-12 is described and related to the effect of valine on resting and growing cells of the three strains.

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MATERIALS AND METHODS

The strains of *E. coli* used included wild-type strains W and K-12 and two derivatives of strain K-12. One was strain K-12/VR, selected for its ability to give rise to colonies on minimal agar supplemented with 50 μg of L-valine per ml. The other was strain 12B14D6, a threonine and leucine auxotroph derived by two mutational steps from strain K-12. The minimal medium and the procedures for growing and harvesting the cells have been described previously (Umbarger and Brown, 1957). After harvesting the cells by centrifugation and washing them twice with buffer, the cell paste was suspended in 8 volumes of 0.05 M potassium phosphate (pH 8.0). The suspensions were disrupted by sonic oscillation in a Raytheon 10-kc magnetostrictive sonic oscillator cooled with water at 1 C. The disrupted suspensions were centrifuged at 28,000 × *g* for 15 min in a Servall SS-1 centrifuge. The sediment was discarded and the clear supernatant fluids were either used immediately or stored at −10 C until needed. The protein content of the extracts, determined by the biuret method of Gornall, Bardawill, and David (1949), was 10 to 15 mg per ml.

Acetolactate was determined as acetoin by the method of Westerfeld (1945) after decarboxylation by heating in the presence of 1.8 N H_2SO_4. Acetohydroxybutyrate was determined by the method previously described (Leavitt and Umbarger, 1960).

For the determination of valine among the amino acids extracted from whole cells by trichloroacetic acid (free amino acid pool), six 2-liter flasks, each containing 400 ml of minimal medium, were inoculated with sufficient early log phase cells to yield an initial optical density of 0.03 at 420 mμ. Growth was measured turbidimetrically using a Klett-Summerson colorimeter with a no. 42 filter against a blank of uninoculated medium. The flasks were incubated with shaking at 37 C. At intervals during the logarithmic and stationary phases of growth, samples were removed and the cells were harvested by centrifugation. The cells were suspended in cold 5% trichloroacetic acid for 30 min, and the extracted amino acids were adsorbed on a Dowex-50 cation-exchange column in the H^+ form. The amino acids were eluted from the column with 4 N NH_4OH, and samples were applied to Whatman no. 1 paper. After chromatography in a *n*-butanol–acetic acid solvent (10:1) saturated with water, the papers were sprayed with a 0.4% solution of ninhydrin in water-saturated butanol. The valine spot was eluted with a solution of 0.005% copper sulfate dissolved in 70% ethanol and the intensity of the colored solution determined (Giri, Radhakrishnan, and Vaidyanathan, 1952).

In the experiments involving the accumulation of α-aminobutyric acid, the cells were centrifuged for 15 min at 28,000 × *g* and the supernatant liquids were desalted by treatment with Dowex-50 as described above. Samples were applied to Whatman no. 1 filter paper and the amino acids were later separated by descending chromatography in *n*-butanol saturated with 2 N NH_4OH. α-Aminobutyrate was identified by comparison with a known standard. The amino acid was determined microbiologically (Leavitt and Umbarger, 1960). The assay organism was strain M97-21, an isoleucine auxotroph of *E. coli* W lacking L-threonine deaminase.

For the studies on the uptake of valine from the medium, membrane filter techniques similar to those described by Britton, Roberts, and French (1955) were used.

RESULTS

Effect of L-valine on an extract of E. coli K-12. By use of an extract prepared from *E. coli* K-12, the effects of valine on acetolactate formation and on acetohydroxybutyrate formation were compared. Both reactions were inhibited by valine to essentially the same extent (Table 1). The specificity of this inhibition is indicated by the fact that both leucine and α-aminobutyrate, at a similar concentration, were inactive.

Reversal of L-valine inhibition by pyruvate. It has previously been demonstrated that valine inhibition of acetolactate formation is competitive with respect to pyruvate (Umbarger and Brown, 1958). Figure 1 shows a similar relationship with respect to acetohydroxybutyrate formation. The addition of increasing amounts of pyruvate reverses valine inhibition in a competitive manner, with α-ketobutyrate kept constant.

Effect of L-valine on extracts of E. coli strains not sensitive to valine. Since it has been postulated than the inability of *E. coli* K-12 to grow in the presence of valine is due to inhibition of aceto-

hydroxybutyrate formation by valine, it was of interest to determine whether or not valine inhibited acetohydroxybutyrate formation in extracts prepared from a strain of *E. coli* that is not inhibited by valine. An extract of the valine-insensitive strain, *E. coli* W, and an extract of the strain K-12 mutant which grew in the presence of valine, were examined. Both acetolactate and acetohydroxybutyrate formation by the extract of *E. coli* W could be inhibited by valine, but this extract was much less sensitive than that of strain K-12. The extract of the valine-resistant mutant of strain K-12 was even less sensitive (Table 2). This correlation suggests that the greater sensitivity of acetohydroxybutyrate formation to valine in strain K-12 is responsible for the ability of valine to inhibit the growth of this organism.

TABLE 1. *Inhibition of acetolactate and acetohydroxybutyrate formation**

Inhibitor (5.0×10^{-5} M)	Acetolactate		Acetohydroxybutyrate†	
	Amount formed	Inhibition	Amount formed	Inhibition
	μmole	%	*μmole*	%
None	0.54	0	0.39	0
L-Valine	0.25	55	0.18	52
L-Leucine	0.54	0	0.39	0
α-Aminobutyrate	0.54	0	0.39	0

* Each tube contained 100 μmoles of potassium phosphate (pH 8.0); 10 μmoles sodium pyruvate; 10 μmoles sodium α-ketobutyrate, where indicated; 80 μg thiamine pyrophosphate; 10 μmoles magnesium chloride; inhibitor where indicated; and crude extract of *E. coli* K-12, containing 4.5 mg of protein; total volume, 1.0 ml. The reaction mixture was incubated in air at 37 C for 15 min. The reaction was stopped with 0.1 ml each of 10% zinc sulfate and 1 N sodium hydroxide.

† Reaction mixture contained 10 μmoles of sodium α-ketobutyrate.

Effect of valine sensitivity on the level of intracellular valine. If the inhibition of acetolactate formation by valine is in fact a feedback control mechanism, the level of valine within the extractable pool should be related to the differences in valine sensitivity observed in extracts. The more sensitive the feedback control, the smaller should be the level of valine maintained within the pool. The valine-insensitive strain of *E. coli* W, when growing exponentially in minimal medium, had five times more valine in its pool than did the valine-sensitive strain K-12. In addition, the valine-resistant mutant of strain K-12, whose enzyme was even less sensitive than that of strain W, had an even higher level of valine in its pool (Fig. 2). The level of valine maintained within the cell thus seems to be inversely related to the sensitivity of acetolactete formation to valine.

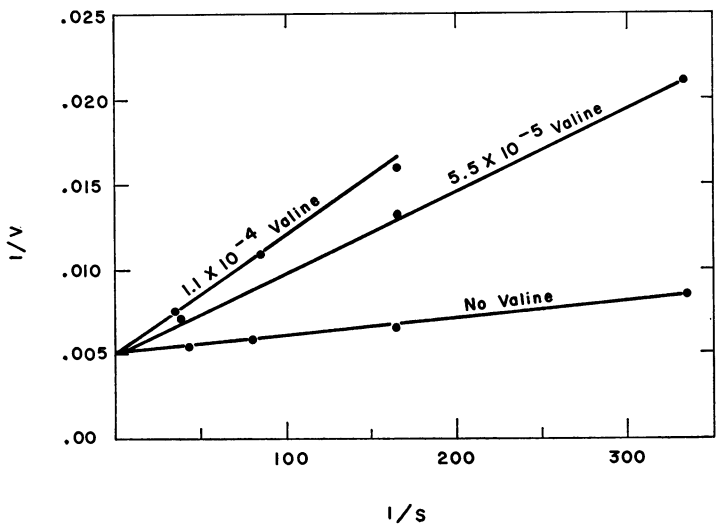

FIG. 1. *Double reciprocal plot showing the effect of valine on the formation of acetohydroxybutyrate. Conditions as described in Table 1. 1/V = reciprocal of μmoles of acetohydroxybutyrate formed in 15 min. 1/S = reciprocal of μmoles of pyruvate used as substrate.*

TABLE 2. *Valine inhibition of acetolactate and acetohydroxybutyrate formation**

Compound formed	Valine	Strain W		Strain K-12		Strain K-12/VR	
		Amount formed	Inhibition	Amount formed	Inhibition	Amount formed	Inhibition
		μmoles	%	*μmoles*	%	*μmoles*	%
Acetolactate	None	0.43	—	0.54	—	0.46	—
	5.0×10^{-5} M	0.39	8	0.25	55	0.46	—
	1.0×10^{-4} M	0.37	14	0.12	78	0.42	8
	2.0×10^{-4} M	0.33	23	0.07	87	0.39	11
Acetohydroxy-butyrate†	None	0.32	—	0.39	—	0.35	—
	5×10^{-5} M	0.30	5	0.20	48	0.35	—
	1×10^{-4} M	0.28	12	0.14	63	0.31	12
	2×10^{-4} M	0.25	21	0.07	82	0.29	15

* Each tube contained 100 μmoles of potassium phosphate (pH 8.0); 10 μmoles sodium pyruvate; 10 μmoles sodium α-ketobutyrate, where indicated; 80 μg thiamine pyrophosphate; 10 μmoles magnesium chloride; L-valine where indicated; and bacterial extract containing 4.5 mg of bacterial extract prepared from *E. coli* strains as indicated; total volume, 1.0 ml. Conditions as in Table 1.

† Reaction mixture contained 10 μmoles of sodium α-ketobutyrate.

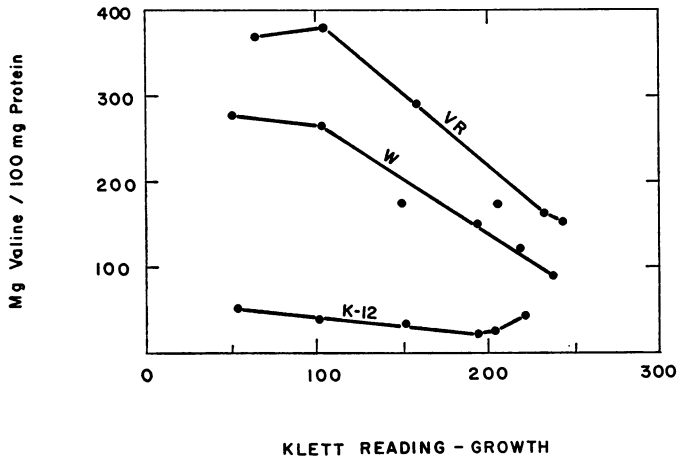

FIG. 2. *Intracellular level of L-valine in growing cells of Escherichia coli strains W, K-12, and K-12/VR. Conditions as described in text.*

The high intracellular levels of valine were maintained in strain W and the valine-resistant mutant of strain K-12 only at relatively low cell densities. As the cultures became more dense, the level of valine within the pool fell sharply, with a concomitant increase in the level of valine in the medium. It may be that the inability of cells to retain endogenously formed valine is brought about by the less aerobic environment obtained at higher cell densities, much as the concentration of exogenously added valine has been found to be sensitive to metabolic inhibitors such as sodium azide and dinitrophenol (Cohen and Rickenberg, 1956).

Accumulation of α-aminobutyrate by strain 12B14D6. In view of the fact that valine inhibits the formation of acetohydroxybutyrate, the addition of valine to the growth medium of the valine-sensitive strain might be expected to cause the accumulation of the compound preceding the blocked reaction, α-ketobutyrate. To examine this possibility, use was made of strain 12B14D6, a threonine and leucine auxotroph derived by two mutational steps from *E. coli* K-12. Since this mutant was unable to synthesize threonine, the threonine within the cell would be derived entirely from the exogenous supply, thus eliminating any effect of the rate of threonine synthesis

which might be influenced by various experimental conditions. In addition, the omission of leucine from the culture medium of the doubly blocked mutant made it possible to demonstrate that the accumulation of α-ketobutyrate, should it occur, would be a result of the inhibition of acetohydroxybutyrate formation by valine rather than the result of the cessation of growth.

Log-phase cells of this organism were grown in a medium supplemented with excess threonine and glycyl-L-leucine. A peptide of leucine was used rather than leucine itself, to eliminate the possibility of leucine and valine competing with one another for entry into the cell. The cells were centrifuged, washed twice in 0.05 M phosphate buffer (pH 8.0), and used to inoculate flasks containing 30 ml of minimal medium supplemented with 200 μg per ml of glycyl-L-leucine, 50 μg per ml of L-valine, and 100 μg per ml of L-threonine. Although an examination of the culture fluids after a 60-min incubation period failed to reveal any differences in the amount of keto acid present, appreciable quantities of its amination product, α-aminobutyrate, appeared in the culture fluid supplemented with threonine, glycyl-L-leucine, and valine (23.0 μg per 100 ml of medium). However, when growth was limited by the omission of glycyl-L-leucine from the medium, rather than the addition of valine, little α-aminobutyrate was detected (4.6 μg per 100 ml of medium). It would thus appear that the accumulation of α-aminobutyrate in the presence of valine is not a fortuitous consequence of cessation of growth, but rather is the result of a specific interference with the synthesis of isoleucine.

Uptake of valine by E. coli strains K-12 and W. The inhibition by valine of the K-12 strain but not of strains W and K-12/VR might well be explained solely on the basis of the differences in sensitivity to L-valine of the respective acetohydroxybutyrate-forming systems. However, the existence of a more sensitive enzyme system does not preclude the possibility that strain K-12 may also be more permeable to valine. The ability of strain W and K-12 to concentrate exogenously added valine was therefore examined to explore this possibility. Early log phase cells of each strain were incubated with valine in 20 ml of minimal salts medium. After equilibrating with shaking for 2 min at 37 C, 3.0-ml samples were removed and the cells separated from the culture fluid by filtration through a membrane filter. The cells were washed with 3.0 ml of cold minimal medium, and the "free pool" was extracted with 5.0 ml of cold 5.0% trichloroacetic acid. When valine was added to exponentially growing cultures, *E. coli* strains W and K-12 accumulated the amino acid to the same degree (Fig. 3).

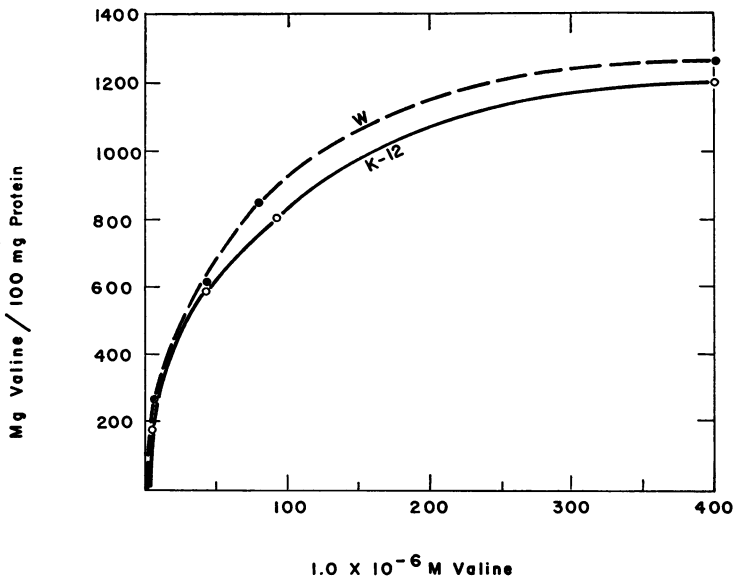

FIG. 3. *Uptake of L-valine by Escherichia coli strains K-12 and W*

DISCUSSION

It would appear from the experiments reported here that inhibition of the growth of *E. coli* K-12 by valine is a consequence of the greater sensitivity to valine, in this strain, of the enzyme system that forms acetohydroxybutyrate. Although this primary site of valine inhibition can be clearly recognized, the possibility is not excluded that, when isoleucine synthesis is thus blocked, other effects of valine may secondarily result.

Indeed, Cohen (1958) called attention to the fact that the addition of valine to an exponentially growing culture of this *E. coli* strain does not cause an abrupt cessation of growth. Rather, the optical density of the culture continues to increase at a linear rate. He attributed the increase to false protein formed by the incorporation of valine in place of isoleucine. Evidence was presented that at least some of the enzymes formed during the period of linear growth were less active, and that the protein formed by the valine-sensitive K-12 strain when grown in the presence of labeled valine had a higher proportion of labeled valine than that formed by the valine-resistant mutant. This finding was interpreted as evidence that the proteins formed by the K-12 strain were abnormal. Another possibility is that Cohen's valine-resistant mutant, like strain K-12/VR described here, had an acetolactate-forming system less effectively inhibited by valine, so that it would be less able to quench the endogenous formation of valine.

Because of the dual role that the acetolactate-acetohydroxybutyrate system plays in biosynthesis, alterations in the sensitivity of this system illustrate especially well the consequences of a cell having enzymes whose function is restricted by such regulatory mechanisms as repression of enzyme synthesis and end-product inhibition of enzyme activity. As has been emphasized earlier, when a single chemical transformation serves two distinct functions, the only way the cell can control the two functions is to develop two enzymes, each geared to its own specific task (Halpern and Umbarger, 1959; Umbarger, 1961). That this development did not occur in the two functions for acetohydroxy acid formation has led to the complication described in the K-12 strain of *E. coli*. It would be interesting to know why a separate enzyme for acetohydroxybutyrate synthesis was not selected during evolution. The reason may be that whenever valine appeared in the environment isoleucine also appeared, so that there has, in fact, been no selection against valine sensitivity. The division of bacterial strains into valine-sensitive and valine-resistant types would then be due to other, unrecognized selection pressures.

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